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Retinal Research using the Perfused Mammalian Eye

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CONTENTS

Abbreviations: BRB, blood–retina barrier; DC ERG, DC coupled electroretinogram; ERG, electroretinogram, recorded from cornea corneous; ONR, optic nerve response: negative, temporally dispersed action potential recorded from the optic nerve as a "straight light" retinal signal; OFF component, response to "light off", action potential of the optic nerve; ON component, response to "light action potential of the optic nerve; OP, oscillatory potentials of the ERG; RPE, retinal pigment epithelium; SP, standing potential vertebrate eye, cornea-positive; STR, scotopic threshold response; V/log I, signal amplitude vs. log stimulus intensity function.

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Abstract—The effort to isolate and maintain alive *in vitro* an intact mammalian eye is rewarded by the full control provided over the arterial input and exclusion of systemic regulatory or compensatory mechanisms. Electrical recording of typical light-evoked field potentials from retina and optic nerve can be complemented by single-cell recording. Thus, light-induced electrical activity reflects the function of the retinal pigment epithelium, of the layers of the retina and of the ganglion cells or their axons. Retinal function *in vitro* is documented by electrophysiological and morphological methods revealing subtle features of retinal information processing as well as optic nerve signals that approach—at threshold stimulus intensity—the human psychophysical threshold. Such sensitivity of third-order retinal neurons is described for the first time. This well controlled *in vitro* preparation has been used successfully for biophysical, metabolic and pharmacological studies. Examples are provided that demonstrate the marked sensibility of the rod system to changes in glucose supply. Moreover, histochemical identification of glycogen stores revealed labeling of the second- and third-order neurons subserving the rod system, in addition to labeling of Müller (glial) cells in the cat retina. The glycogen content of the cat retina is augmented by prolonged anesthesia, largely depleted by ischemia after enucleation and enhanced by insulin. Pharmacological experiments using agonists and antagonists of putative retinal neurotransmitters are summarized and outlined using the muscarinic cholinergic agonist QNB as an example. Actions and uptake of the neuromodulator adenosine are presented in detail, including inhibitory effects on physiologically characterized ganglion cells. Neuronal effects of adenosine are distinguished from those resulting from vasodilatation and from glycogenolysis induced by the neuromodulator. To open the blood–retina barrier, a hyperosmotic challenge can be applied transiently. This process is monitored histochemically using FITC-albumin and with electrophysiological parameters. Changes in vitreo–scleral resistance and in the amplitude of the EOG-light peak appear to reflect the open/closed status of the barrier. This overview of the uses of the isolated perfused mammalian eye in retinal research concludes with a discussion of potential implications for clinically relevant topics. © 2001 Elsevier Science Ltd. All rights reserved

1. BACKGROUND AND OBJECTIVES

The purpose of this article is to present and discuss an intact mammalian eye preparation and its use in the context of pharmacological, metabolic and functional morphology studies. Beginning with methodological aspects including practical hints and pitfalls, I will summarize evidence establishing the functional and morphological integrity of the retina *in vitro*. I will discuss effects of changes in biophysical parameters, anatomical and physiological correlations, effects of metabolic challenges, studies of neurotransmitter actions and controlled, transient opening of the blood–retina barrier (BRB). Evidence arising from this work for non-neural contributions to the generation of the b-wave, and the value of the *in vitro* approach to address clinically relevant issues round off this overview. The results presented are necessarily a selection of data from several laboratories with emphasis on observations made by the author and his collaborators.

The primary advantages for perfusing mammalian eyes *in vitro* despite the very involved technique (Fig. 1) comprise: (i) control over the chemical input to the retina, while excluding systemic regulatory mechanisms and recycling, (ii) maintaining the retinal integrity with the choroid, retinal pigment epithelium (RPE), optic nerve and vascular system, (iii) an opportunity to exactly control arterial concentration and timing of externally applied pharmacological agents or metabolic substances, (iv) access to retinal electrophysiological parameters at several levels of information processing while simultaneously recording the optic nerve action potential, as sketched in Fig. 11a.

The work of Gouras (Gouras and Hoff, 1970; Fig. 3) provided the foundation for this technique which several investigators have adapted, modified and refined over three decades, thereby extending the range of meaningful applications.

There is ongoing controversy and interest to study the multiple contributors to the generation of the b-wave of the ERG. In an isolated eye with the retina remaining in its natural environment of adjacent structures, metabolic, biophysical and pharmacological parameters can be varied in a precisely controlled manner. The possibility to change only one single variable at a time allows study of the factors that contribute to the generation of the b-wave, an inherently complex field potential. Moreover, a *purely neural* field potential, the light-evoked optic nerve response (ONR) can be compared to the b-wave under various challenges that often yield diverging changes (Section 3.8). The threshold of the ONR has been recorded *in vitro* for the first time approximately one log unit *below* the scotopic threshold response (STR), the most sensitive ERG component encountered. The ONR turned out to be excellently suited to study subtle pharmacological effects as well as the action of exogenously

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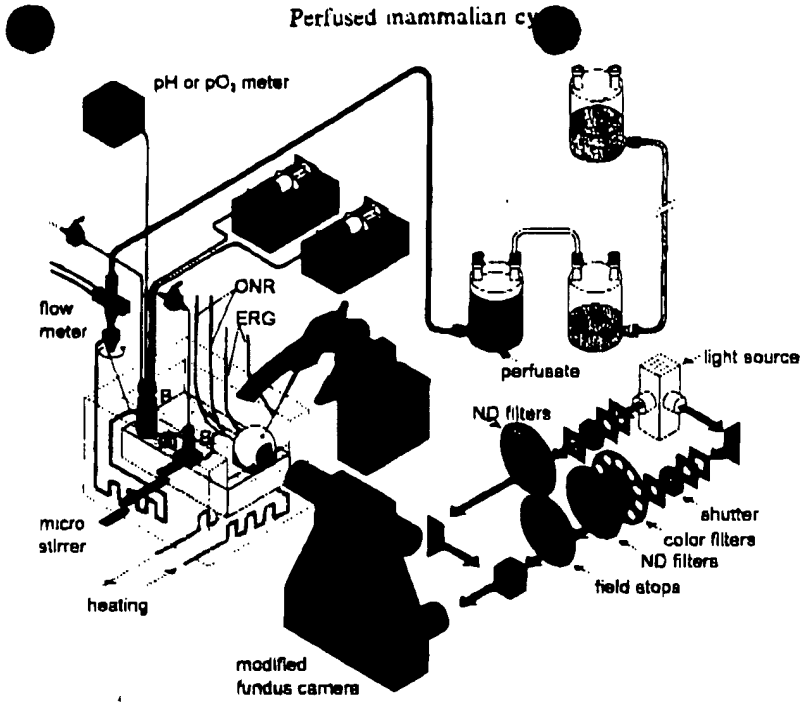

Fig. 1. Scheme of setup developed for perfusion of mammalian eyes (not to scale). Hydrostatic pressure (right upper corner) drives the perfusate (gray) through a drop count-chamber and a stainless-steel multiturn valve mounted below. The perfusate passes through two bubble trap chambers (B), that can be vented (1-way valves). Substances injected by one or more pump-driven syringes are mixed with the perfusate at the outflow channel of the larger bubble trap chamber. To ensure optimal mixing a microstirrer is mounted at the smaller bubble trap chamber near the ophthalmociliary artery. The outflow of perfusate is removed by suction (not shown) from the smaller chamber that holds the isolated eye. The position of the recording electrodes is shown in Fig. 2.

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Changes in flow-rate of perfusion reflect changes in vascular resistance, as observed during most pharmacological manipulations. These effects have to be subtracted from the observed effects of agents as predictable changes that would be expected from an increase or decrease in flow of perfusion alone. We therefore generated transient changes in flow-rate of the magnitude observed under drug action to assess these effects separately. Due to constraints in space, I will not consider the studies on aqueous humor dynamics (Macri, 1960), perfusate flow rate and autoregulation (Papst *et al.*, 1982), and separation of choroidal from global ocular blood flow (Yu *et al.*, 1988).

A typical pharmacological experiment or a metabolic challenge, referred to as a "series" follows a standard protocol, beginning at least 1 hour after onset of perfusion to allow time for dark-adaptation as well as stabilization of the *in vitro* status of the isolated eye. Light-evoked responses from the retina and optic nerve are recorded to evaluate stability and serve as controls. Then the agent under study is applied via a pump-driven syringe for the appropriate time period (e.g. 10 min), and changes in flow-rate (corresponding to change in total vascular resistance of the eye), the standing potential (SP), the ERG and the ONR are measured. Washout begins at the termination of injection (without recycling), and 30–60 min of recovery are allowed prior to the next series with a higher concentration of the drug or with another substance.

2. METHODS AND PRACTICAL HINTS

Different investigators use a variety of approaches to prepare and maintain animals and isolated eyes for electrophysiological study *in vitro*. I shall outline the actual technique used currently in our Neurophysiology Laboratory at the University of Zürich (Fig. 1).

The experiments were conducted in accordance with the resolution on the use of animals in research of the "Association for research in vision

and ophthalmology" and, with the regulations of the cantonal veterinary authority of Zürich. Details on the method have been published previously (Gouras and Hoff, 1970; Niemeyer, 1973a, 1975, 1981, 1992; Niemeyer and Gouras, 1973a,b; Jurklies *et al.*, 1996; Kaelin-Lang *et al.*, 1999).

2.1. Surgery and perfusion

After premedication with atropine sulfate (0.02–0.04 mg/kg, s.c.), animals were anesthetized first with an injection of ketamine hydrochloride (Ketalar, 10–20 mg/kg i.m.; Graeub, Bern/Switzerland) and then with pentobarbital hydrochloride (Nembutal, 9–16 mg/kg i.v.; Abbott Laboratory, Chicago, USA). Intubation was done after injection of gallamine triethiodide i.v. as a bolus and subsequent continuous infusion (Flaxedil, 5–10 mg/kg). Deep anesthesia was maintained by continuous pump-driven i.v. application of pentobarbital. The electrocardiogram, the transcutaneous oxygen saturation and the expiratory CO_2 were monitored continuously, and a respiration pump (model 66IA, Harvard Apparatus, South Natick, USA) was used for artificial ventilation with oxygen-enriched (30%) room air for up to 12 h when necessary. Blood oxygen saturation above 94% end-tidal expiratory CO_2 of 3–4% were maintained throughout anesthesia. Rectal temperature (37–39°C) was regulated by a heating pad. Sodium chloride infusion (0.9%, 10 ml/kg/h) was used to maintain fluid- and electrolyte balance. Fentanyl (0.05 mg bolus i.v.; Janssen, Baar, Switzerland) was applied prior to each surgical procedure to ensure analgesia. Eyes were enucleated after atropine- and phenylephrine-induced mydriasis and under anticoagulation (Liquemin, 1000–2000 U, i.v. bolus; Roche Pharma, Basel, Switzerland). A corresponding amount of protamine hydrochloride (1000–2000 U, Roche Pharma, Basel, Switzerland) was injected after the enucleation of the first eye. Animals were sacrificed after enucleation of the second eye with an overdose of Nembutal. The ophthalmociliary artery was prepared under an operating microscope assessing shunting arterial branches, and the intactness of the long posterior ciliary arteries. Visible arterial shunting branches were cauterized at this point or after onset of perfusion. After

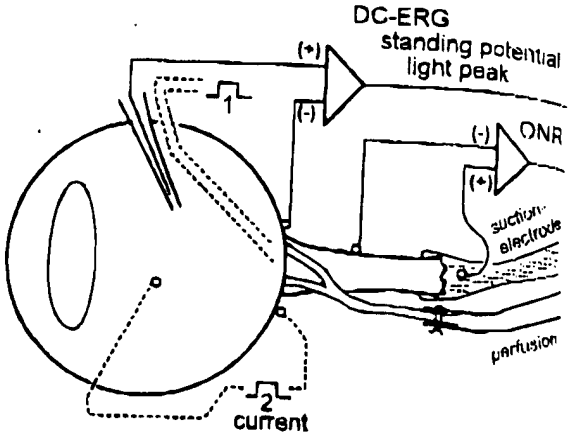
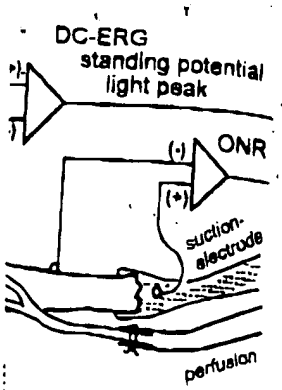


Fig. 2. Position of the recording electrodes for the ERG and ONR and of the AgAgCl electrodes for electrical stimulation (not to scale). (1) A pair of preretinal electrodes to stimulate the optic nerve electrically (Section 3.1; Fig. 5) and electrodes (current 2) to pass current pulses across the retina/RPE/choroid/sclera (Section 3.7). Note the polarity of the ONR preamplifier to display the signals as negative upwards. Corneal contact lens electrode not shown.

cannulation of the ophthalmociliary artery (Figs. 1 and 2), the eyes were perfused with oxygenated tissue culture medium (TC 199, Bioconcept, Allschwil, Switzerland) with Earle's salts, L-glutamine (200 nM), amikacin-sulfate (63.9 µM, Amikin, Bristol-Meyers Squibb AG, Baar, Switzerland) and newborn calf serum (30% v/v). The perfusate was buffered with HEPES (15 mM) and $NaHCO_3$ (26 mM) to a pH of 7.4 at 37°C and gassed with humidified oxycarbon (95% O_2, 5% CO_2) for 25 min to reach a pO_2 of 400–450 mm Hg. The flow-rate was continuously recorded via an infrared drop-interval meter with an analog voltage output. Constant hydrostatic pressure drove the perfusion system, such that changes in flow rate reflected changes in the total vascular resistance of the eye. In order to avoid (i) an increase in intraocular pressure due to fast flow rates of the perfusate and (ii) for insertion of a fine glass pipette carrying the silk-wick AgAgCl electrode (Niemeyer and Kueng, 1999) into the vitreous, a small pars plana vitrectomy was performed at 12 o'clock at a 7–8 mm limbus distance following moderate cauterization of that region. The procedure facilitates optimal control of the perfusion pressure of the retina and helps to avoid major changes in flow rate as occurring e.g.



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during osmotic challenges intended to open the blood–retina barrier (see Section 3.7).

2.2. Stimulation and recording

The source for the light stimuli was a 150 W xenon arc lamp providing a maximum of 11.54 log quanta [scot., 507 nm] $deg^{-2}s^{-1}$ at the eye (see scale in Fig. 6). The stimuli were applied in Maxwellian view via a modified funduscamera (Funkhouser and Niemeyer, 1982), or a back-illuminated diffusor in front of the isolated eye in DC-ERG-light peak experiments. Attenuation of the light flashes was performed with neutral density filters and narrow-band chromatic filters to achieve rod-matched conditions for short- and long wavelength light pulses in full dark adaptation. The shutter-controlled duration of the stimulus was between 20 and 400 ms, and the interval was 5–60 s depending on light intensity and on averaging.

The ERG and standing potential (SP) were recorded between an AgAgCl contact lens—or the intravitreal AgAgCl-silk electrode with an identical reference electrode on the sclera near the optic nerve (Fig. 2).

The light-evoked compound action potential of the optic nerve (optic nerve response, ONR) was recorded with an AgAgCl suction electrode at the cut end of the nerve and with an AgAgCl reference electrode on the surface of the optic nerve, amplified (PARC 1113; Princeton, NJ, USA, band-pass filtered from 0.03 to 100 Hz, 12 dB/octave (variable electronic Butterworth filter, Krohn-Hite 3750, Avon, MA, USA) and fed to a digital oscilloscope (Gould 4050, Cleveland, OH, USA). The ONR was registered, at an expanded time-scale, on a slow multichannel chart recorder (Gould RS 3400, Cleveland, OH, USA) using the analog outputs of the digital oscilloscope. The filtered signals and the flow parameter were also fed into a computer system for data analysis and storage. This system consisted of a personal computer and A/D converter (LabPC+; National Instruments, Austin, TX, USA) and software created with "Labview for windows" (Kaelin-Lang and Niemeyer, 1995; upgraded by P.A. Knapp, MSc, Alea solutions GmbH, Zürich, Switzerland). It allows on-line viewing of changes in amplitudes of the ONR components, of the ERG, of the flow rate, a trend analysis, as well as off-line analysis and plotting of the data. Pharmacologically induced changes in the amplitude of three ONR components were analyzed and compared to control: the ONR-ON component (i.e. the maximal amplitude after light-onset), the plateau, and the ONR-OFF component. The shape of the OFF-component of the ONR varies somewhat between preparations and depends largely on the setting of the low cut-off frequency (Niemeyer, 1981). It can therefore be difficult to quantify the changes in the OFF component induced by the various agents. The ON component, in contrast, has a stable shape at a given stimulus intensity. The ERG b-wave and the standing potential of the eye were also recorded routinely.

Resistance measurements: a constant current plus generator (VCC 600, Physiologic Instruments, San Diego, CA, USA) was used to record the voltage changes induced by vitreo–scleral pulses applied via a pair of AgAgCl electrodes. The procedure worked best with a Teflon-insulated vitreal electrode that was introduced after local cauterization through the pars plana at a 7.5 mm distance from the limbus.

At the beginning of the electrophysiological recordings, immediately after onset of perfusion, standard broad-band red light pulses, 20 ms in duration were applied at 30–60 s intervals during at least 60 min to monitor dark-adaptation and *in vitro* steady-state responsiveness. If the ERG b-wave failed to reach a steady-state amplitude of 600–800 μV, indicating inadequate perfusion, the eye was not used for pharmacological trials.

2.3. Similar preparations used in other laboratories

Other investigators used modified techniques and/or other species, as summarized in Table 1.

2.3.1. *Problems - pitfalls - troubleshooting*

To *avoid bacterial or fungal infection* it is imperative to follow a rigid routine of cleaning the perfusion system as well as the frit for gassing after each experiment and to leave it filled with 70% ethanol between experiments. We use 7 x solution (ICN Biomedicals Inc, Aurora, OH, USA)

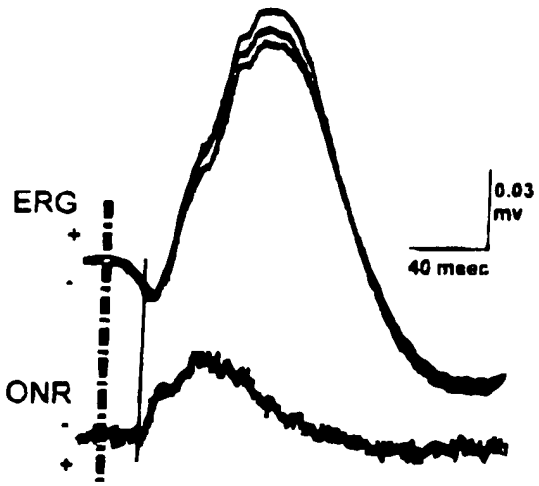

Fig. 3. Historic illustration published three decades ago depicting the first ERG (top trace) and ONR (bottom trace) recordings from a perfused cat eye. The broken vertical line indicates the position of the light stimulus, and 20 ms long light pulse (modified from Gouras and Hoff, 1970; © by permission of the Association for Research in Vision and Ophthalmology).

as the only detergent. If bacterial contamination should occur, the initially normal light-evoked signals decline within 1–2 h, because of rapidly spreading bacterial embolization of the retinal capillaries (Niemeyer and Remé, unpublished).

Extraocular vascular leaks. In the initial phase of perfusion, substantial shunting due to feeding arterial branches on the optic nerve can reduce the supply of perfusate to the preparation, resulting in P III-dominated ERGs with small b-waves but often surprisingly well-maintained optic nerve responses.

An explanation for the variation in optimal flow rates among preparations emerged from scanning electron microscopy on vascular casts of perfusion-fixed cat eyes with identification of arteries and a venous plexus at the surface of the cat optic nerve (Motti and Niemeyer, 1983). We identified a large number of small arterial and venous branches that necessarily and to variable extent are severed during enucleation. Therefore, arterial perfusion of isolated mammalian eyes is expected *a priori* to occur with variable vascular leakage. Because the perfusate appears colorless in the small vessels, leaks are difficult to identify under the dissecting microscope, but once identified can be cauterized. Perfusion is often improved when the vortex vein at the equator on the lower surface of the cat eye is freed (R. Nelson, pers. comm.). Preparing an opening in the pars plana for a small posterior vitrectomy reduces intraocular pressure, thus improving retinal perfusion pressure.

Leaks in the pars plana opening prepared for vitreal recording, however, cannot be identified easily, and subtle cauterization of the tissue around the opening is carried out routinely. Overperfusion can induce cystic changes in the RPE and choriocapillaris, developing into initially very small, but progressive areas of retinal detachment (see Section 3.3).

Table 1. Isolated (*in vitro*) mammalian eye preparations

Species	Preparation	Reference
Cat	Arterially perfused eye	Gouras and Hoff (1970)
	Arterially perfused eye	Niemeyer (1973b)
	Arterially perfused eye cup	Nelson *et al.* (1975)
	Arterially perfused eye	Schneider and Zrenner (1985)
	Arterially perfused eye	Alder *et al.* (1986)
	Arterially perfused eye	Sandberg *et al.* (1987)
	Arterially perfused eye	Thoreson and Purple (1989)
	Perfused eye cup/retinal slices	Peachey *et al.* (1993)
Rat	Arterially perfused eye	Freed and Nelson (1994)
Squirrel	Arterially perfused eye cup	Su *et al.* (1995)
Dog	Arterially perfused eye	Charlton and Leeper (1985)
Rabbit	Arterially perfused eye	Niemeyer (1983)
Cow	Isolated blood-perfused bovine eye	de Monasterio (1978)
Guinea pig	Arterially perfused eye	Tazawa and Seaman (1972)
Monkey	Arterially perfused eye	Cringle *et al.* (1997)
		Schuurmans and Zrenner (1981)

Acid base balance. Careful adjustment of the pH as well as the oxygen partial pressure contributes to the success of an experiment (see Section 3.2). Unsuspected and potentially unnoticed drift of a pH meter and/or pH electrode can lead to changes in the pH of the perfusate resulting in "unexplainably" small b-wave amplitudes, in spite of evidence for technically perfect perfusion.

3. RESULTS

A brief overview necessarily requires a selection of topics, which implies that many studies of equal importance cannot be referred to. The following cited results have in common, that the isolated perfused eye is a preparation suitable for well-controlled short-term experiments. These include preparing for special anatomical fixation procedures, well-controlled metabolic challenges without confounding extraocular compensatory influences, and pharmacological investigations where effects are expected to occur within a few minutes to hours. The stability of responses for over 12 h suffices for repeated pre-drug, drug effect-, and post-drug phases. In the following, I will briefly allude to electrophysiological parameters used to assess retinal function *in vitro* including field potentials and single cell response, address differences *in vitro* and *in vivo* preparations, and summarize specific studies from fellow investigators as well as published and unpublished data from our laboratory.

3.1. Retinal function in vitro

It is essential to test the physiological function and survival of isolated mammalian eyes *in vitro* for periods adequate for the various experiments. We routinely record the pertinent parameters (flow rate of the perfusate, SP, ERG, ONR) on a multichannel chart (in analogy to a tachograph or flight recorder). These recordings help in explaining off-line some counterintuitive reactions to pharmacological or metabolic manipulation: changes in flow rate of the perfusate, in SP, or in amplitudes of steady-state light-evoked responses may indicate irregularities in flow rate or changes in pupil size. Using bright or relatively long stimuli requires corresponding adaptation. After bleaching, the regeneration of rhodopsin has been shown to correspond to the regeneration recorded *in vivo* (Ripps *et al.*, 1989).

Recovery from "low glucose challenge" *in vitro* (Macaluso *et al.*, 1992a) was found to be comparable to that observed *in vivo* (Hirsch-Hoffmann and Niemeyer, 1993; Hirsch-Hoffmann, 1992).

The function of the RPE, outer retina, inner retina and optic nerve are monitored according to the priorities in a given study: the SP, the DC ERG with a-, b-, c-waves and oscillatory potentials, the light peak, the exquisitely sensitive inner retina scotopic threshold response (STR) and the optic nerve action potential. Amplitudes and also the configuration of the ERG components including OPs, the c-wave, and the STR recorded from perfused eyes match their counterparts *in vivo* (Sieving *et al.*, 1986).

Recordings from single cells have been obtained at the National Eye Institute, NIH (Niemeyer and Gouras, 1973a,b; Nelson, 1977), as well as in our laboratory. Whereas RPE cells, horizontal cells and other neurons of the inner nuclear layer require recording with intracellular microelectrodes, recording of the spike activity from ganglion cells with extracellular microelectrodes appears to be more stable over the time needed to study drug effects.

Direct comparison of b-wave amplitudes with those recorded *in vivo* is difficult due to several factors: shunting of the b-wave by the low resistance orbital structures surrounding the globe is expected *in vivo*. In addition, the position of the reference electrode on the forehead *in vivo* and on the sclera *in vitro* introduces major differences. Cringle and colleagues (Cringle *et al.*, 1988) systematically assessed *in vitro/in vivo* differences in the dog. "Supernormal" b-wave amplitudes were recorded at a limbus distance of the scleral reference electrode of 10 mm, and a limbus distance-related gradient of the b-wave of 90 μV/mm was found (Cringle and Alder, 1988). The authors established the existence of isopotential lines around the globe perpendicular to its optical axis. The implicit time decreased with increasing distance from the limbus.

c-wave. Recording the c-wave allows the viability of the RPE to be assessed (Niemeyer, 1976a,b),

which is particularly meaningful in toxicological studies. It is optimal to use a stimulus duration of 4 s to saturate and reliably measure the response at a given light intensity.

A useful test for the functional state of the RPE *in vitro*, in addition to recording the c-wave is to elicit the light peak, an unusually slow component frequently regarded as part of the dc-ERG (Niemeyer and Steinberg, 1984; Niemeyer, 1986a; Steinberg *et al.*, 1987). The light peak recorded from the perfused cat eye is indistinguishable from that recorded in the anesthetized cat (Steinberg *et al.*, 1980; Steinberg and Niemeyer, 1981). It corresponds to the clinically recorded light rise in the EOG (Arden *et al.*, 1962).

3.1. Optic nerve action potential

The accessibility of the optic nerve lends itself to monitor the purely neural output of retinal information. The optic nerve action potential, ONR, allows three distinct components to be distinguished if the stimulus duration is 200 ms or longer (Niemeyer, 1989b): a negative ON component, a plateau phase and a more variable OFF component. The components following the very stable and reproducible ON component greatly depend on the time constant of the amplifiers/filters, on adaptational state, and also on the wavelength of the stimulus (Fig. 4). Schuurmans and Zrenner (1980, 1981) identified the two cone mechanisms with spectral sensitivity maxima at 460 and 560 nm in the perfused cat eye, as used and confirmed in a study on effects of adrenergic agonists (Uji *et al.*, 1988; Miyamura and Uji, 1993).

To further characterize optic nerve activity *in vitro*, we looked for conduction velocities of the major groups of ganglion cells. Electrical stimulation was applied to the disc (Fig. 2) and responses were recorded with the electrode arrangement that is used for the light-evoked ONR. Hypothermia to 34°C was used to demonstrate the two groups of conduction velocities that correspond to the axons of X- and Y-ganglion cells, respectively (Fig. 5; Rowe and Stone, 1976; Niemeyer, 1976a,b).

3.1.2. *Dark-adapted threshold*

We were interested in assessing the threshold of the light intensity that reproducibly generates electrical field potentials in the fully dark-adapted perfused cat eye. Determining thresholds provides insight regarding *in vivo* and *in vitro* function in order to document pharmacologically or genetically induced changes in retinal sensitivity (Niemeyer, 1988, 1989b, 1991a,b, 1995, 1997a, 1998; Kueng-Hitz *et al.*, 1999a,b). Figs. 4 and 6 depict the b-wave threshold and the rod-driven scotopic threshold response, STR, a signal with potential clinical relevance. The action potential of the optic nerve, ONR, under full dark-adaptation consists at threshold stimulus intensity of relatively slow, late (latency > 100 ms) and negative ON- and OFF-components without the plateau that appears only at higher stimulus intensities (Fig. 4). We performed experiments for measuring the threshold intensities of several signals in full dark adaptation: the lowest intensities necessary to elicit the b-wave, the STR, and the ONR are compiled in Fig. 6. For comparison, we added *in vivo* recorded electrophysiologic threshold data as well as the intensity determined for the human psychophysical threshold.

Interestingly, the OFF component was often found to exceed the ON component in amplitude at threshold stimulus intensity. This particular effect could be reversed by applying a very dim background (illumination below 0.1 lux). The "enhanced OFF-response" was independent of stimulus duration. Any prior light adaptation ("light history"), however, appeared to prevent the OFF-component from exceeding the ON-component in amplitude.

Averaging obviously "cleans" the ONR traces recorded near threshold, but prevents detection of fluctuations in the amplitude of the signal. In preliminary experiments, fluctuation in the amplitude of ONRs near threshold was observed in several isolated eye preparations. The frequency of this fluctuation appears to be irregular and therefore different from that described for the human psychophysical threshold (Ripps and Weale, 1976).

Comparing the light intensity used here with *in vivo* data from other laboratories (Robson and Frishman, 1999; Sieving and Wakabayashi, 1991) showed good agreement with respect to thresholds for the b-wave and for the STR. The ONR thresholds reflecting activity of third-order

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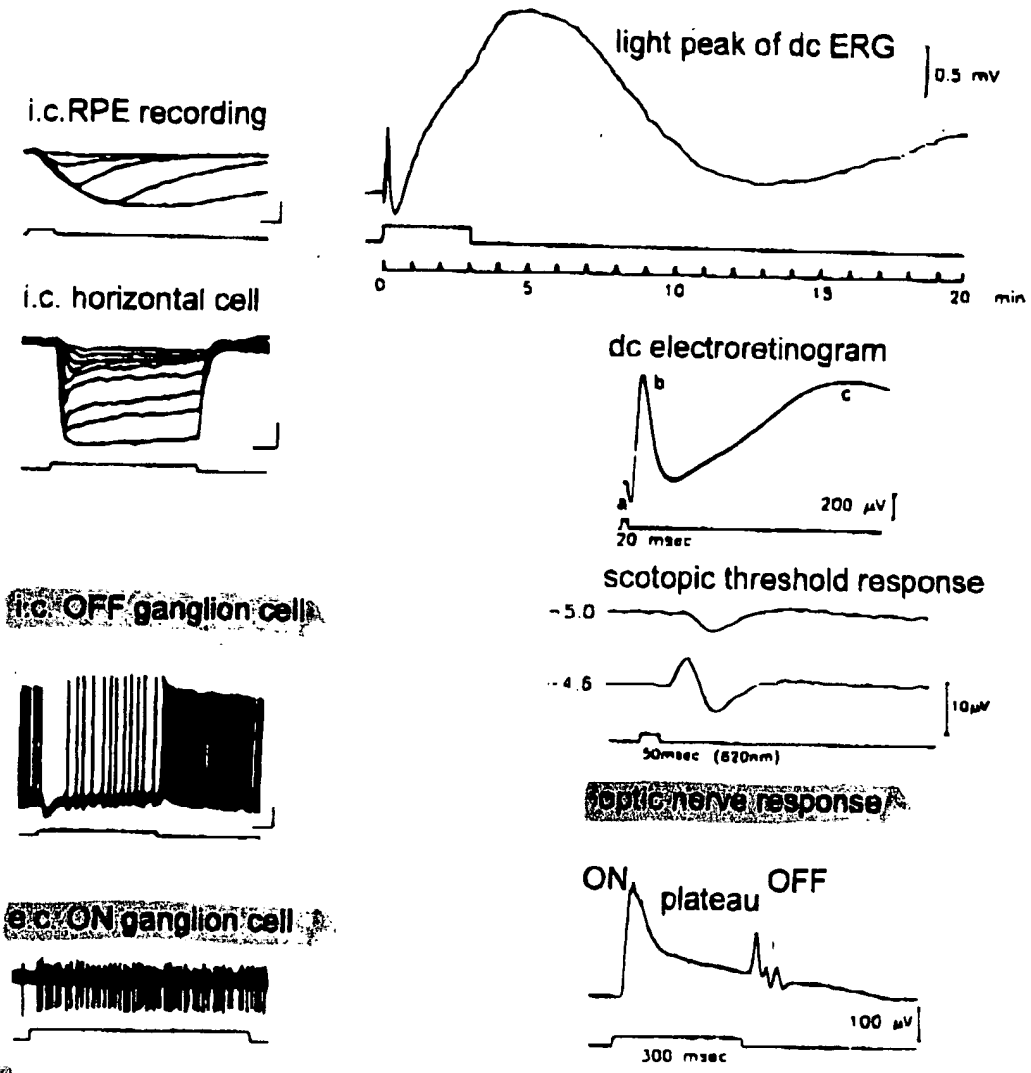

Fig. 4. Composite of typical light-evoked signals from the perfused cat eye. Left, single-cell responses to stimuli of increasing intensity recorded intracellularly (i.c.; 5 mv calibration bars) and extracellularly from an ON-center ganglion cell. Right, the slow light peak of the DC electroretinogram (top), the ERG at a slow time scale to display the vitreally recorded c-wave, the STR at two intensities (note the intrusion of the b-wave at relative intensity "4.6 ND filter"). A typical optic nerve response recorded in dark-adaptation, about 3 log units above threshold is shown in the lowermost trace (modified from Niemeyer, 1992).

neurons *in vitro*, to our knowledge presented here for the first time, closely approach the human psychophysical threshold of vision (Finkelstein and Gouras, 1969; Frishman *et al.*, 1996; Aguilar and Stiles, 1954).

Sensitivity of the retina is reflected in greater detail in V/log I functions. They are useful in a variety of pharmacological investigations (Fulton, 1991; Niemeyer, 1991a,b). The V/log I functions are stable over several hours (Niemeyer, 1975), and ERG as well as ONR recordings can be obtained for up to 12 h. It was found that the threshold sensitivity of physiologically identified X- and Y-type ganglion cells in the dark-adapted perfused cat eye (Niemeyer *et al.*, 1991) was comparable to that in the anesthetized cat (L. Frishman, pers. comm.).

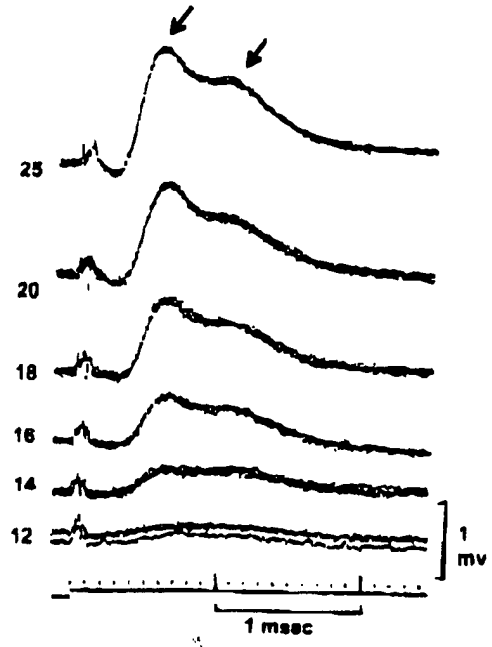

Fig. 5. Electrically evoked responses from the optic nerve *in vitro*, revealing the two major conduction velocity groups (arrows; Rowe and Stone, 1976). Electrical stimulation of the disc (see Fig. 2) with pulses of 0.1 ms at increasing voltages as indicated to the left of the superimposed traces. The speed of the responses is slowed down by hypothermia in this experiment (34°C).

3.2. Changing biophysical parameters

An isolated central nervous system preparation needs a finely tuned continuous supply of nutrients. To satisfy these requirements, Ames III designed a type of heart-lung machine for the isolated rabbit retina with excellent results (Ames III and Zager, 1987). We followed Gouras and Hoff (1970) with arterial perfusion of the isolated eye, where the critical factors comprise adequate flow rate of the perfusate to supply oxygen and glucose as well as to remove metabolic waste, adequate pH and pCO_2, all at a temperature of 37.5°C, independent of the flow rate. We attempted to reduce these requirements to a practical protocol of perfusion that maintains the isolated eye preparation in a physiologic state for more than 10 h using serum-enriched medium TC 199-based perfusate with physically dissolved oxygen and buffered with HEPES and bicarbonate. Higher flow rates were shown to compensate for a lower oxygen content revealing autoregulation of ocular vasculature (Papst *et al.*, 1982) to an extent comparable to retinal autoregulation in the anesthetized cat (Niemeyer *et al.*, 1982).

Another approach to adapt the *in vitro* to the *in vivo* condition was used by Alder *et al.* (1986). vitreal (preretinal) oxygen tension gradients were recorded at carefully monitored distances from the retina or retinal vessels. This study revealed similar profiles of oxygen distribution *in vitro* and in the anesthetized animal.

The vasodilatating effect of additional bicarbonate improves perfusion (Papst *et al.*, 1982; Winkler, 1972). ERG changes during stepwise hypothermia revealed a linear increase in b-wave amplitude between 28 and 38°C (Niemeyer, 1975). Temperature constancy (37.5°C) at varying flow rate was achieved by directing the perfusate in glass tubes through a large thermostatic water bath with heat exchange coils. The retina as a highly metabolic tissue was tested extensively for changes in its functional properties with shifts in intracellular pH (pH_i). Changes in pH of the perfusate (pH_o) *in vitro* only induced effects when the cytoplasmic pH was affected (pH_i). "Intracellular pH" was used as a conceptual term since intraretinal or intracellular pH was not actually measured. Preretinal measurements of pH during induced acid–base changes, however, yielded evidence for corresponding changes in intraretinal pH (Niemeyer and Weingart, 1986).

Hypercapnia *in vivo* was shown to decrease b-wave amplitude (Niemeyer *et al.*, 1982; Linsenmeier *et al.*, 1983). Various ways to change the pH of the perfusate (pH_o) in the isolated cat eye by intraarterial injection of an inorganic chemical or by substitution with another perfusate with high or low pCO_2 allowed us to conclude that a decrease in pH_i induced a decrease in b-wave amplitude and in the amplitude of the light peak, a slow light-induced rise of the DC-ERG. Increase in pH_i induced an increase in b-wave amplitude but left the light peak essentially unaffected. The vascular resistance, reflected inversely in the flow rate of the perfusate (under constant pressure) consistently decreased under acidification, and increased under alkalization. In short, the b-wave of the ERG increases with alkalization and decreases with acidification.

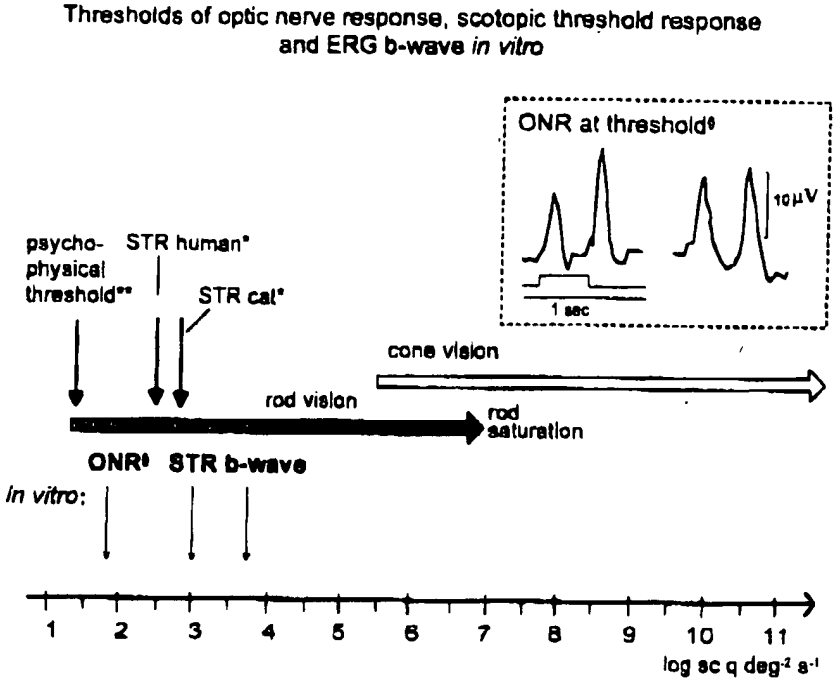

Fig. 6. Presentation of threshold data *in vivo* (thick arrows) and *in vitro* recorded data (thin arrows). The psychophysical threshold value ** is taken from Finkelstein and Gouras (1969) and STR data * are from Robson and Frishman (1999). The intensity scale is relative to the maximal output of the xenon source in our laboratory, the maximum unattenuated output being 11.5 log scotopic quanta/deg²/s measured at the position of the cornea of the isolated eye. Inset: two examples of averaged ($n = 4$) ONRs at threshold, often presenting with larger OFF than ON amplitudes. §Optic nerve response recorded *in vitro*.

The glucose content of the standard perfusate (5.5 mm) corresponding to that in cat serum turned out to be at the lower limit for function of the rod system. The marked effects of changes in glucose supply will be described in detail in Section 5.2.

3.3. Retinal function and anatomy

In this Section I will discuss selected studies that assessed the structural integrity of isolated perfused eyes under controllable modification of the metabolic supply *in vitro* and studies that specifically capitalized on the advantages of the preparation to identify physiologically characterized neurons with intracellular dye injections followed by elaborate morphological reconstruction.

Early on it was clear that the influence of changing the flow rate of perfusion upon retinal ultrastructure must be assessed. Increasing the flow rate produced saturation of the b-wave amplitude, with variation between preparations (Niemeyer, 1973a,b). The effects of low, medium and deliberately high (above normal) perfusion flow rates were then studied electrophysiologically and morphologically at the light- and electron microscopic levels (Remé and Niemeyer, 1975). The expected electrophysiological consequences were as expected: traces with only the P III component at low flow rates, normal b-waves at medium flow rates, and enhanced ERG waveforms at high flow rates of perfusion. The corresponding morphological picture revealed specific changes compared to the controls, which were specimens from eyes fixated 8 min following enucleation without perfusion. At high flow rates patches of cystic changes in the RPE were observed that may initiate multiple retinal detachments as seen frequently after more than 10 h of perfusion (unpublished observation). Cell counts in electron microscopy specimens revealed increasing percentages of slight and severe cellular changes in inverse proportion to the flow rate of perfusion. Interestingly, of all cell types

the Müller cells were the most sensitive in the inner nuclear layer, an observation with implications for the mechanism of generation of the ERG b-wave (Section 3.8).

Perfusion fixation of isolated eyes proved to be a suitable tool for particular anatomical studies (Rungger-Brändle *et al.*, 1993) and identification of cell-specific distribution of glycogen stores (Rungger-Brändle *et al.*, 1996). In addition, the glycogen content could be assessed under different metabolic conditions (Niemeyer *et al.*, 1997; Lansel *et al.*, 2000; see also Section 4.3) .

A modification of the intact isolated eye preparation is the isolated perfused eye cup developed by Nelson (1977). In a number of publications Nelson and coworkers employed a powerful association of techniques combining intracellular recording of multiple biophysical response characteristics with subsequent structural identification of cell types and synaptic connections. Removing the cornea, lens and vitreous facilitates the use of micropipette electrodes to record intracellularly from neurons as small as a cone (Nelson, 1977), leading to detailed structure–function analysis also of horizontal, bipolar, amacrine and ganglion cells. The now established functional and structural segregation of ON- and OFF-sublaminae in the inner plexiform layer emerged from these morphological studies combined with sophisticated physiology (Nelson *et al.*, 1976, 1978). In sum, this approach contributed greatly to our understanding of the circuitry of the mammalian retina (for a comprehensive review, see Kolb and Nelson, 1984; see Fig. 10 in Kolb, 1994).

3.4. Metabolic challenges

In developing the technique of perfusing isolated cat eyes we chose the glucose content of the perfusate intuitively according to the normal range of serum glucose in cats (see Dawis *et al.* (1985) for details of perfusate composition). Normal serum glucose levels in various mammalian species are compiled by Macaluso *et al.* (1992a). Selectively testing the effects of small changes in glucose concentration is feasible in arterially perfused preparations, while keeping all other variables constant.

Whereas many investigators use media with markedly elevated glucose concentrations for various *in vitro* preparations, the standard tissue culture medium TC 199 contains 5.5 mM glucose. Glucose contents of perfusates for various *in vitro* preparations are listed in Onoe and Niemeyer (1992). The concentration of 5.5 mM matches the species-specific serum glucose level of the cat. However, the results of a detailed study employing the perfused eye led to the conclusion that the supply of glucose (5.5 mM) in the dark-adapted mammalian retina must be considered at the lower limit (Macaluso *et al.*, 1992a). The isolated perfused eye allows the supply of glucose to be controlled precisely, while avoiding extraocular regulation that could confound data analysis. We used rod-driving and, with a white background, cone-driving stimulation and recorded ERG b-wave, scotopic threshold response, optic nerve response, standing potential and c-waves.

Microelectrode recordings from the subretinal space separated transretinal from trans-RPE c-wave components (Macaluso *et al.*, 1992a). We implemented step changes in glucose concentration for 5–8 min, derived V/log I curves near threshold, and measured DC-standing potential recordings. Figure 7 depicts glucose-dependent changes in rod- and cone-driven b-waves in comparison to corresponding changes in the ONR-ON component (Figs. 2 and 4 in Onoe and Niemeyer, 1992). A striking result was the marked sensitivity to changes in glucose concentration of the rod system compared to robust cone responses, which decayed and eventually vanished only under extreme hypoglycemia. A second feature is the larger extent of changes in the ERG b-wave compared to the smaller extent of the changes in the ONR (see also differences between b-wave and ONR in Section 3.8). Another clear-cut difference between the rod-driven b-wave and ONR was found in latency changes: low glucose dose-dependently prolonged the latency of the b-wave, whereas the latency of the ONR did not change significantly (Lansel and Niemeyer, unpublished observation).

When eyes were perfused *a priori* with a higher concentration of glucose (8 or 10 mM), additional glucose had *no* or only *minimal* effects on rod-driven ERG and ONR-signals (Onoe and Nie-

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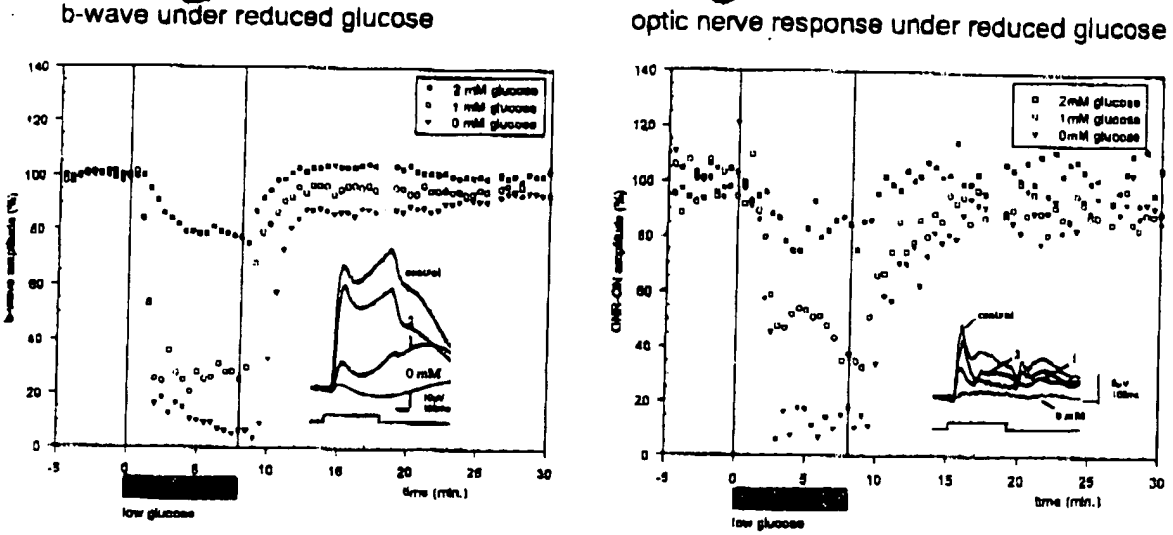

Fig. 7. Effects of reducing the supply of glucose in the perfusate on the rod-driven b-wave and on the ONR. Glucose concentrations were changed from the (normal) 5.5 mM level to 2, 1 or 0 mM for 8 min. The effects were dose-dependent and reversible within < 30 min and were more pronounced for the ERG b-wave than for the ONR. Inset: samples of traces before and during low glucose. Full dark-adaptation and stimulation with pulses about 1 log unit above threshold, 400 ms in duration. Error bars represent 1SD, $n = 2$.

meyer, 1992). We interpret this observation as evidence for the glucose levels in the cat's serum being just barely sufficient to subserve the function of the rod system. The purely rod-driven STR was affected by changes in glucose concentration much like the synchronously recorded ONR. Cone-driven signals recorded in presence of a light adapting, rod-desensitizing background, in contrast, revealed no sensitivity to changes in glucose supply, unless levels were below about 2 mM.

Electrical activity of the pigment epithelium, monitored as the "resting" or standing potential (SP), as well as the c-wave of the DC-ERG also exhibited marked sensitivity to changes in glucose. The SP increased and decreased by maximally 2.5 to 3 mV in parallel with the increase and decrease of glucose concentration, respectively. These changes were observed in the dark- as well as in the light-adapted state. The RPE, a glia-like structure, thus responded to changes in glucose concentration independently of rod- or cone stimulation. The c-wave, mainly rod driven, decreased and increased in amplitude much like the SP, but with greater variability. By recording from the subretinal space with microelectrodes, we identified the RPE-component of the c-wave as opposed to the P III-component as being responsible for the glucose-induced changes in the vitreal c-wave (Fig. 10 in Macaluso *et al.*, 1992a).

In spite of ample evidence supporting the notion that the perfused cat eye exhibits retinal physiology that matches *in vivo* responses (Section 3.1), confirming glucose sensitivity *in vivo* represented a challenge. A protocol for glucose clamping in the anesthetized cat with quantitative ERG recordings in the fully dark-adapted as well as in the light-adapted state was developed (Hirsch-Hoffmann, 1992; Hirsch-Hoffmann and Niemeyer, 1993). Briefly, long-term general anesthesia with intubation and artificial ventilation, monitoring of arterial blood pressure, central venous sampling for frequent determination of glucose levels, application of somatostatin to block endogenous production of insulin, and infusion of insulin extended the anesthesia protocol outlined in Section 2. The corneal ERG was recorded using Ganzfeld flash stimulation (PS 22 flash; Astro-Med, West Warwick, RI, USA) in full dark adaptation at an intensity of about 1.5 log units above the threshold of the b-wave for rod-driven signals, and employing Ganzfeld-light adaptation (320 mW/m^2) for cone-driven signals.

The results obtained *in vivo* indeed revealed a marked sensitivity of the rod-driven b-wave to

glucose-infusion-induced hyperglycemia and to insulin-induced hypoglycemia. The resulting increase and decrease in serum glucose (occurring much more slowly) was followed by an increase and decrease in b-wave amplitude, respectively. A rapid decay of the b-wave amplitude appeared when glucose levels fell below 1.8–2 mM (Fig. 3 in Hirsch-Hoffmann and Niemeyer, 1993). Corresponding cone-driven ERGs showed no or small changes, with a decrease in glucose below 1.5 mM inducing increases in the cone-driven b-wave in some cases. Thus, the normalized rod- and cone-b-wave data plotted vs. glucose levels revealed a picture essentially matching that obtained *in vitro*. To answer whether the rod–photoreceptor mass potential, P III, with its fast and slow components was affected by changes in serum glucose, we isolated the signal by intravitreal injection of aspartate *in vivo*. Because of the resulting blockage of synaptic transmission to second order neurons, the b-wave declined and disappeared within 1.5–2.5 h. The isolated P III component of the ERG failed to change when serum glucose was increased or decreased (Fig. 7 in Hirsch-Hoffmann and Niemeyer, 1993). The rod-driven receptor potential thus appeared to be largely independent of the glucose level of the circulating blood.

3.4.1. *Cellular distribution and content of glycogen in the cat retina*

Two projects to enhance the understanding of the role of glycogen stores in the cat retina were realized in our laboratory: first, it appeared meaningful to assess the cellular distribution of glycogen in the cat retina; second, the quantity of glycogen stored in the retina was measured in dissected retinas under specific *in vivo* and *in vitro* conditions.

The first project required histochemical identification at the electron microscopic level of glycogen as beta particles of 10–20 nm in diameter or as clumped densities of about 50 nm in diameter. We also attempted to identify the neurons that contained glycogen in juxtaposition to the Müller cell's ramifications (Rungger-Brändle *et al.*, 1996).

We used both arterial perfusion fixation of isolated eyes and immersion fixation prior to preparing the samples for histochemistry and visualization of glycogen in thin sections. The major surprise in the results was that in addition to homogeneous distribution of particulate glycogen in Müller cells, selected neurons also contained glycogen: the two major classes of ganglion cells, the α- and β-types revealed heavy content of glycogen, and rod bipolars and A17 as well as A22 amacrine cells contained particulate glycogen. However, photoreceptors and cone bipolar cells were free of glycogen. Glycogen thus appeared as a marker not only for Müller cells, but also for second- and third-order neurons that subserve the rod-driven circuit. This is documented in Rungger-Brändle *et al.* (1996) and illustrated in the examples in Fig. 8. Müller cells in the periphery contained more diffusely distributed glycogen than those in the central retina. The distribution has been described to be quite different in the anangiotic rabbit retina (Magalhäs and Coimbra, 1970; Matschinsky, 1970).

To assess the content of glycogen quantitatively, we dissected retinas from deeply anesthetized cats and from *in vitro* perfused eyes under controlled and specifically modified conditions. We dissected retinas immediately after enucleation following short- or long-lasting anesthesia. Retinas were also dissected from eyes 10 min after enucleation – imitating the time span prior to perfusion in the *in vitro* experiments. Retinas were dissected under dim red light and snap frozen in liquid nitrogen. The retinal glycogen content was measured after enzymatic treatment using sonication, drying, exposure to amyloglycosidase in triethanolamine, and the final concentration was measured fluorimetrically with the values expressed as µg glucose (converted from glycogen) per mg protein (Niemeyer *et al.*, 1997). The content of glycogen in cats increased with increasing duration of the barbiturate anesthesia (see Section 2): data obtained after 1.5 h compared to > 10 h of anesthesia are shown in Fig. 9. This phenomenon has been observed before (Phelps, 1972). The question arose which measurement would be a suitable reference for the *in vitro* experiments. We chose to use the glycogen content just prior to perfusion *in vitro*, that is, 10 min after enucleation (see Section 2) at room temperature as the "starting" or reference value (third column from left in Fig. 9). Depriva-

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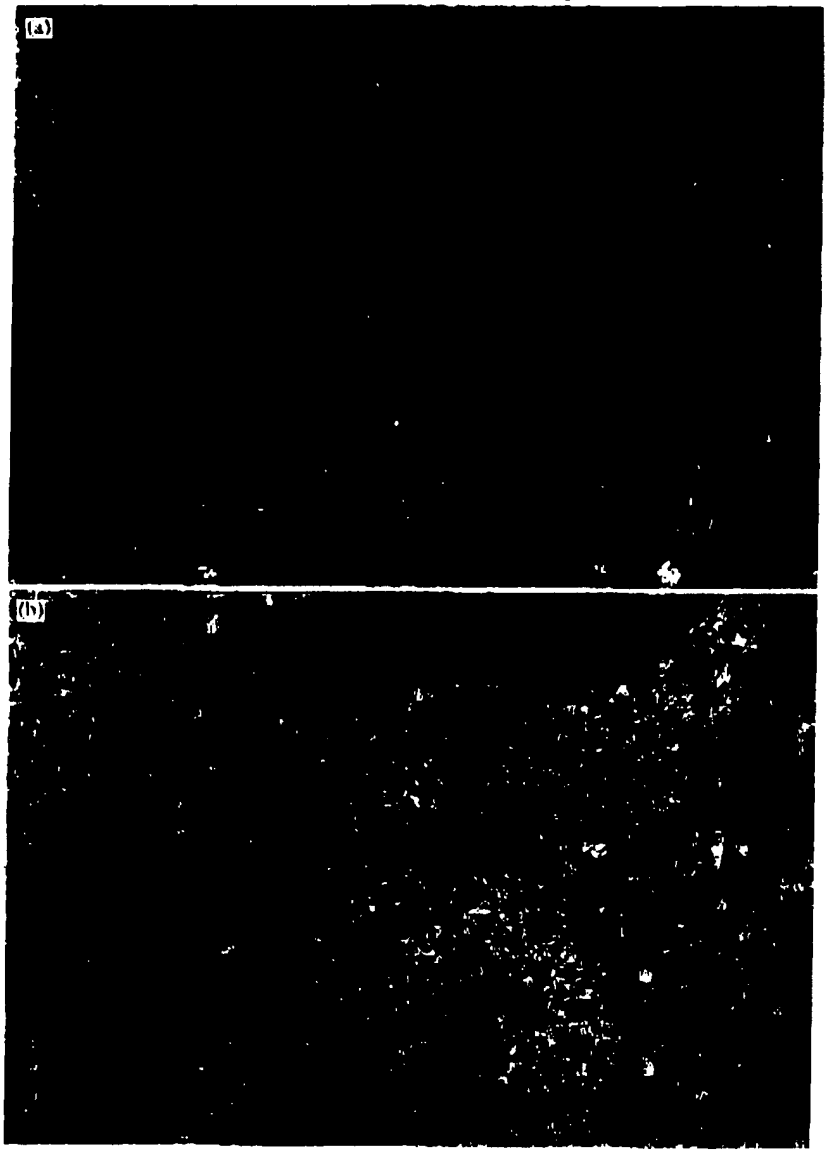

Fig. 8. Glycogen particles in the cat retina shown in electronmicrographs. Middle and inner retina oriented such that the vitreal side = bottom of the figure. (a) inner portion of the inner nuclear layer and inner plexiform layer revealing clumped glycogen in an amacrine cell (A) and dispersed glycogen granules in the cytoplasm of Müller (M) and rod bipolar (RB) cells. (b) dispersed glycogen granules in two ganglion cells (G). Bars represent 1 μm. For details see Rungger-Brändle *et al.* (1996). (Reproduced from Niemeyer, 1997b; © by permission from Wiley-Liss, Inc., a subsidery of John Wiley & Sons, Inc, New York).

tion of glucose using perfusate with zero glucose for as long as 80 min resulted in a rapid decrease and disappearance of the light-evoked responses ERG and ONR within <30 min. Much to our surprise, the glycogen content was decreased but not depleted after 80 min under this extreme condition.

Perfusion with "normal" (5.5 mM) glucose concentration for 3 h only moderately increased glycogen content *in vitro*. Perfusion in this way but with insulin added, however, restored glycogen to the *in vivo* level after 1.5 h of anesthesia. Similar levels were reached when eyes were perfused for >12 h under "normal" conditions in spite of

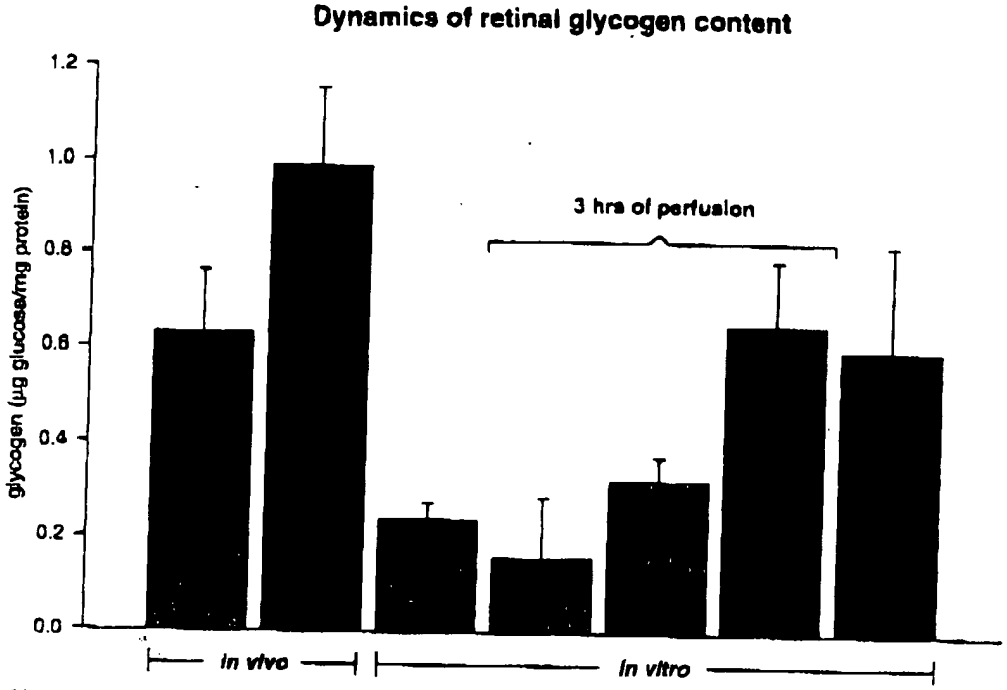

Fig. 9. Glycogen content of the retinas of cat eyes from anesthetized animals and from *in vitro* preparations. Retinas were dissected and snap-frozen after treatment according to the conditions indicated in the columns, and stored in liquid nitrogen prior to subsequent analysis. Anesthesia was pentobarbital hydrochloride based, as indicated in Section 2. Insulin was applied intraarterially at a 10 × postprandial concentration. In the last column (right) "brief low glucose challenges" refer to experimental series of the type illustrated in Fig. 7. Error bars represent 1SD, $n = 2$.

repeated brief (10 min) low glucose challenges of the type shown in Fig. 7. These low glucose challenges, however, induced marked and reversible decrease in the amplitudes of the light-evoked electrical signals.

To investigate effects of insulin on retinal glycogen stores isolated eyes were perfused under the following conditions: normoglycemic perfusion for 3 h, normoglycemic perfusion plus insulin (140 µunits/ml, corresponding to 10 × postprandial level) for 3 h (Lansel *et al.*, 2000). Insulin markedly increased the retinal glycogen content without effects on light-evoked electrophysiologic signals.

3.4.2. *Insulin*

We examined short-term effects of insulin compared to postprandial increase *in vivo* in experiments on the perfused eye. Multiple roles of insulin in the central nervous system as well as its presence in the retina along with its degrading enzyme, insulin transhydrogenase have been shown (summarized in Lansel and Niemeyer, 1997). Insulin-free perfusate was enriched with albumin instead of calf serum in these experiments. Effects of postprandial, and 10–20 times higher concentrations of insulin, applied arterially were different with normal compared to low glucose conditions. Insulin failed to affect the rod-driven b-wave and ONR. The SP increased consistently and dose-dependently by up to 0.75 mV, suggestive of interactions with insulin-receptors in the RPE.

Under low glucose conditions (a brief step-decrease in glucose concentration), insulin enhanced the extent of the decrease in b-wave amplitude produced by low glucose, but left the extent of the decrease in ONR amplitude practically unchanged. This effect was similar for insulin concentrations from 5 to 100 ng/ml insulin. We consistently observed an increase in the latency of the b-wave, but no change in timing of the ONR under insulin (Lansel and Niemeyer, unpublished

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Insulin plus low glucose: different changes in b-wave and ONR

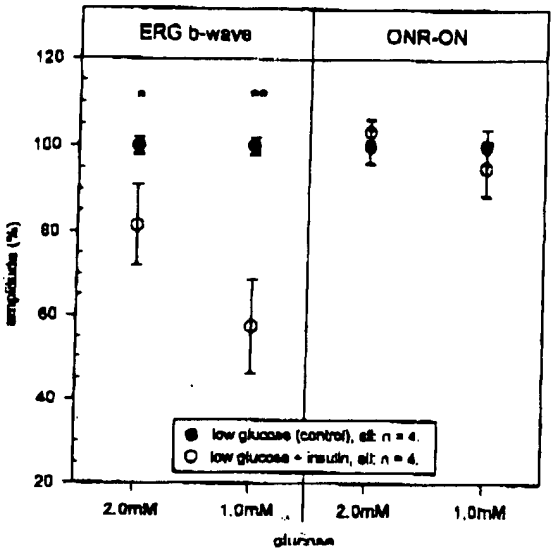

Fig. 10. Low glucose challenges and insulin. Effects of reducing glucose concentration to 2 or to 1 mM, respectively, were examined in dark-adapted perfused cat eyes *per se* (filled circles, $n = 4$ experiments) or in combination with insulin (5 ng/ml, empty circles, $n = 4$ experiments). The reduction in response amplitudes during reduced glucose was set to 100%. Insulin induced an additional decrease in b-wave amplitude, but not in the ONR amplitude. Means of 4 experiments; ±SEM (from Lansel and Niemeyer, 1997; © by permission of the Association for Research in Vision and Ophthalmology).

observation). Our results support the concept of a saturable transport mechanism of insulin across the BRB (Fig. 10).

It was concluded that insulin apparently is not required for maintaining retinal function in a perfused cat eye over 10 h and more. The differential effect of insulin under low glucose points towards changes in the glial contribution to the ERG-b-wave without changing the purely neural ONR. The insulin-enhanced reduction of b-wave amplitude under low glucose was interpreted as a suppression of the use of glucose by Müller cells.

3.5. Neurotransmitters

Studies of pharmacological effects on mammalian retina can aim to understand mechanisms of drug action or can be designed to primarily elucidate retinal function (Niemeyer, 1991b, 1988; Schneider and Zrenner, 1986; Schuurmans and Zrenner, 1983). Intraarterial application, in well controlled nanomolar to micromolar concentrations of agonists or antagonists of retinal neurotransmitters allow pharmacological effects to be studied, provided that the substances cross the BRB. Washout is guaranteed after termination of the injection of the substance into the perfusion, and the duration of the effects reflect the kinetics of the receptor binding, without extraocular influences such as recycling or metabolizing actions. Of the light-evoked signals that reflect the different layers of retinal information processing, the optic nerve action potential, ONR, proved the most subtle pharmacological indicator with great reproducibility (Niemeyer, 1989b; Jurklies *et al.*, 1996; Kaelin-Lang *et al.*, 1999). Analysis typically includes response intensity functions for the ON component, often also for the plateau- and OFF component of ONRs obtained under rod- or cone-driving stimulus conditions. Changes in amplitude and configuration of the ONR reflect summed and inherently complex changes in the proportion of excitation and inhibition in the different retinal ganglion cells. The field potential thus yields information on sensitivity to, and dose range of, pharmacological compounds. Specific effects are preferably studied on single isolated retina cells and at the level of single ion channels. When interpreting results of pharmacological experiments in the isolated eye preparation we consider the passage of a substance across the BRB, across the vitreo-retinal interface, potential changes in perfusate flow, effects on single neurons and glia, and finally their assumed influence on the field potentials.

Table 2 contains information on selected studies that addressed neurotransmitter-, neuromodulator- and drug-related issues employing perfused mammalian eye preparations. The interested reader can find direction and details that cannot be dealt with in this article in previous reviews (Uji and Niemeyer, 1989; Niemeyer *et al.*, 1981; Niemeyer, 1991b).

As an example, typical dose-dependent and reversible changes in the configuration of the ONR were elicited by quinuclidinyl benzilate

Table 2. Overview of pharmacological mainly transmitter-related studies in perfused, isolated eyes

Title (by theme)	References
Cholinergic	
Cholinergic effects on cat retina *in vitro*: changes in rod- and cone-driven b-wave and optic nerve response	Jurklies *et al.* (1996)
Binding and electrophysiology of the muscarinic antagonist QNB in mammalian retina	Niemeyer *et al.* (1995)
Transmitter-related studies in the isolated, perfused eye of the cat	Niemeyer *et al.* (1981)
Effects of atropine on ERG and optic nerve response in the cat	Niemeyer and Cervetto (1
β- adrenergic	
Influence of buphenine on the retina of the isolated mammalian eye	Cottier *et al.* (1986)
Effects of buphenine (nylidrin) on the perfused mammalian eye	Niemeyer *et al.* (1987b)
The perfused mammalian eye	Uji and Niemeyer (1989)
β-adrenergic antagonists modify retinal function in the perfused cat eye	Gerber and Niemeyer (198
Effects of β-adrenergic antagonists on rod-mediated retinal function in the perfused cat eye	Niemeyer *et al.* (1988)
The effects of β-adrenergic agonists on cone systems in the cat eye	Uji *et al.* (1988)
Catecholaminergic binding sites in cat retina, pigment epithelium and choroid	Bruinink *et al.* (1986)
Effects of clenbuterol, a β_2-adrenergic agonist, in the perfused cat eye	Gerber and Niemeyer (198
Effects of β-agonists on b- and c-waves implicit for adrenergic mechanisms in cat retina	Niemeyer *et al.* (1987a)
Adenosine	
Adenosine modulates physiology in the perfused cat eye	Fruch *et al.* (1990)
Adenosine: autoradiographic localization and electrophysiologic effects in the cat retina	Blazynski *et al.* (1989)
Effects of adenosinergic agents on the vascular resistance and on the light-	Kurz-Lang *et al.* (1999)
Adenosine inhibits antimuscarinic light-evoked activity in x- and y-ganglion cells in the perfused cat eye	Niemeyer *et al.* (1991)
Monoamines, GABA and other agents	
Similarity and diversity of monoamines in their effects on the standing potential, light peak and electroretinogram of the perfused cat eye	Dawis and Niemeyer (1988)
Theophylline alkaloides the light peak in perfused cat eyes	Dawis and Niemeyer (1987)
Dopamine influences the light peak in the perfused mammalian eye	Dawis and Niemeyer (1984)
Effects of D-1 and D-2 dopamine antagonists on ERG and optic nerve response of the cat	Schneider and Zrenner (1981)
The effect of fluphenazine on rod-mediated retinal responses	Schneider and Zrenner (1987)
Vincristine-induced changes in the retina of the isolated arterially perfused cat eye	Ripps *et al.* (1989)
Functional role of GABA in cat retina: II. Effects of $GABA_A$ antagonists	Frumkes *et al.* (1995)
Effects of IBMX on the ERG of the isolated perfused cat eye	Sandberg *et al.* (1987)
The influence of phosphodiesterase inhibitors on ERG and ONR of the cat	Schneider and Zrenner (1986)
A role of the angiotensin–renin system for retinal neurotransmission?	Zrenner *et al.* (1989)

(QNB), a ligand of muscarinic acetylcholine receptors, by strychnine, an antagonist of glycine, by bicuculline, an antagonist of γ-amino-butyric acid and by clenbuterol, a selective β_2-adrenergic agonist (Niemeyer, 1989b). Cholinergic agonists increased the plateau component of the ONR, whereas cholinergic antagonists produced the opposite effect. Thus, QNB a muscarinic antago-

used, isolated eyes

References

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Cholinergic agonists
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nist with high density of binding sites in the cat retina, induced marked, dose-dependent andreversible effects on the ONR (Niemeyer *et al.*, 1995).

Drug effects on field potentials can be examined at the single cell level, e.g. in recordings from horizontal cells or extracellularly from ganglion cells (Niemeyer *et al.*, 1991). Recording the graded S-potentials from horizontal cells intracellularly allows us to document or to exclude action of a neurotransmitter-related agent in the outer retina and thereby to analyze contributions to the retinal field potentials. Using this approach we could show that atropine sulfate, mecamylamine and dihydro-beta-erythroidine failed to affect horizontal cells in the perfused cat eye (Niemeyer *et al.*, 1981; Niemeyer, 1986b). The data indicate that these cholinergic antagonists have no action at the outer retinal level of information processing.

3.6. Neuromodulation by adenosine

Adenosine, a neuromodulating purinergic nucleoside is ubiquitously distributed in the CNS, and its functional role has been reviewed in general physiology (Williams, 1990; Stone, 1991) and for the retina (Blazynski and Perez, 1991; Kaelin-Lang *et al.*, 1999). The uptake of ^{3}H adenosine in the cat retina revealed localization in various layers of the mammalian retina. We demonstrated autoradiographic localization of uptake of labeled adenosine (Blazynski *et al.*, 1989), revealing distinct labeling over the inner nuclear layer and over many cells in the ganglion cell layer (which can include displaced amacrine cells in addition to ganglion cells) as well as intense perivascular labeling. Uptake by RPE cells can be observed unequivocally in longer exposed retinal sections in Fig. 11b (Niemeyer, Frishman, Blazynski, unpublished; see also Friedman *et al.*, 1989).

The spectrum of *functional* implications for adenosine accordingly comprises vascular metabolic, glial and neuronal effects. Figure 11a shows a simplified sketch of the mammalian retina in which the sites of effects of adenosine (right) are indicated in relation to the sites of generation of light-evoked electrical potentials (left). The uptake of adenosine in the RPE was found to correlate well with functional results showing that external adenosine increases the SP and the amplitude of the c-wave, but reduces the light peak of the DC-ERG (Frueh *et al.*, 1989). Looking at more proximal layers of the retina, a marked enhancement of the rod-driven b-wave, but an enhancement followed by a reduction of the cone-driven b-wave was observed. To exclude the possibility that the vasodilatation-induced increase in perfusate flow alone was responsible for the increases in b-wave amplitude we tested the effects of comparable flow increases separately. The adenosine-induced enhancement of the b-wave always exceeded that induced by an increase in flow alone. It is likely that the increase in glycogenolysis (Magistretti *et al.*, 1986; Osborne, 1989) contributed to this change in the ERG, based on the observations on controlled changes in glucose supply to the cat retina *in vitro* and *in vivo* (see Section 3.4).

The optic nerve action potential, showed dose-dependent inhibition by adenosine and some of its agonists. This effect was recorded consistently in spite of the accompanying increases in flow rate of the perfusate. The agonists used were cyclohexyl adenosine (Niemeyer and Frueh, 1989) and, in a recent study (Kaelin-Lang *et al.*, 1999), the A_1 receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA) and the A_{2a} receptor agonist CGS 21680C (Ciba Geigy, Summit, New York). Experiments employing specific antagonists led to the interpretation that the depressant effects of adenosine on the ONR are A_1- and probably A_{2a}- receptor-mediated. Much like the ONR the rod-driven inner retinal signal STR decreased dose-dependently and reversibly with 4–10 μM intraarterial adenosine (Macaluso *et al.*, 1992b). This effect can also be related to the uptake of labeled adenosine in neurons of the inner part of the inner nuclear layer and in the ganglion cell layer.

A related study of neuroprotective effects of adenosine with potential clinical implications was carried out by Larsen and Osborne (2000). The authors demonstrated in rats, by electroretinography and by monitoring changes in calretinin immunoreactivity, that adenosine can reduce ischemic damage exerted by a controlled increase in intraocular pressure. Li and Roth (1999) recently documented a role for adenosine in

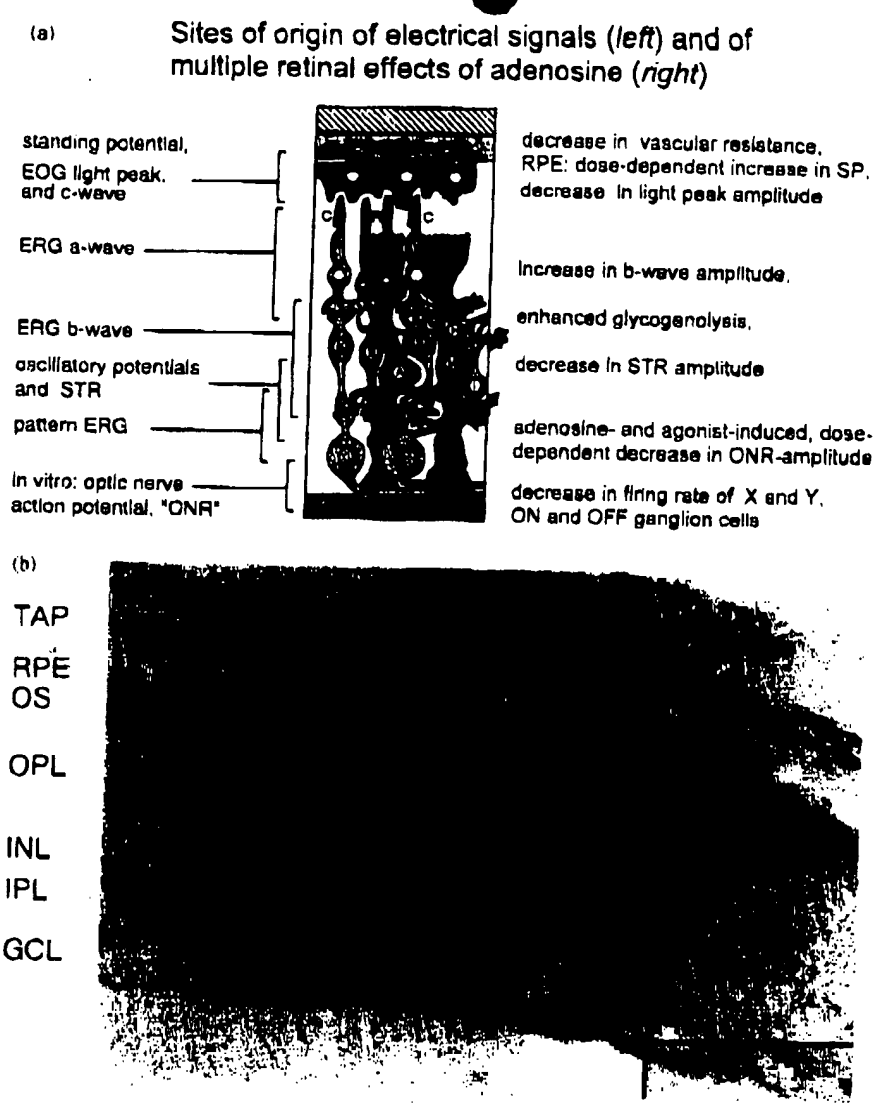

Fig. 11. Scheme of the retina and autoradiogram of labeled adenosine. (a) Effects of exogenous adenosine and approximate sites of origin of the standing potential and of the light-evoked electrical signals. (b) autoradiographic demonstration of uptake of ^{3}H adenosine in the cat eye to show the accumulation of silver grains over the retinal pigment epithelium, RPE (unpublished, obtained in collaboration with Dr. C. Blazynski). In addition, strong labeling of perivascular cells, labeling of some cells in the ganglion cell layer and of some in the inner nuclear layer can be seen as well. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer, OPL, outer plexiform layer; OS, outer segment of photoreceptor cells; TAP, tapetum lucidum — the reflective material of the cat eye between RPE and choroid. Scale bar: 25 μm.

protection of the rat retina from ischemic damage by preconditioning using a selection of specific agonists and antagonists of adenosine (Li and Roth, 1999).

Therapeutic application of adenosine in patients with acute ischemic conditions of the CNS is largely limited by the adenosine-induced decrease in systemic blood pressure (Ribeiro, 1991).

To analyze the marked action of adenosine on the ONR in greater detail, we examined the effect on single retinal ganglion cells of adenosine at concentrations known to modify the ERG and the ONR. We tried to answer the following questions: (1) whether and to what extent the ganglion cell's responses to light are affected by adenosine, (2) whether both spontaneous and light-evoked activ-

ities are affected, and (3) whether effects are confined to subclasses of ganglion cells. A technique was applied that allows rapid physiological classification of ganglion cells: small light stimuli, adjustable in size and position, and the background illumination were produced with the system described (Section 2). The X-Y classification of ganglion cells was based on the linear (X cells) or non-linear (Y cells) spatial summation properties of their receptive fields (Dr. L. Frishman, pers. comm., "method of null position", Enroth-Cugell and Robson, 1966). Briefly, the two halves of a bipartite light stimulus are sinusoidally modulated in counterphase, so that the total light flux does not change over time. When the bipartite field was positioned so that the mean illumination over the entire receptive field remained constant, X cells did not signal the local changes in illumination ("null position"). Y cells, on the other hand, fired at twice the frequency of modulation of the bipartite field when it was similarly centered. These responses were at least half the amplitude of the response when the bipartite field was positioned to maximally stimulate the receptive field center. The bipartite field (60% contrast) had approximately the same mean illumination as the adapting background, that was turned off during the classification test.

Glass micropipettes for extracellular recordings were pulled (Flaming-Brown model P-87, Sutter Instruments Co., CA), beveled, and filled with 2 M potassium acetate, resulting in an impedance of 15–30 MΩ. A microstepper, mounted on an arc-shaped micromanipulator with the center of rotation on the surface of the sclera at the 12 o'clock position, guided the micropipette through the pars plana via a modified needle to the retinal ganglion cell layer. The extracellularly recorded action potentials were amplified (Axoprobe-1A, Axon Instruments, CA, USA), converted to TTL pulses (Window Discriminator Mod.120, World Precision Instruments, CT, USA), and counted within temporal bin widths of 50 ms (Nicolet 1170, Wisconsin) to generate pulse count histograms.

Figure 12 depicts typical histograms of the major classes of retinal ganglion cells that responded to low micromolar concentrations of adenosine. Out of 13 cells studied with sufficient stability and characterized physiologically as ON- and OFF-, X- and Y types, 10 responded to adenosine in much the same manner: consistent and reversible decrease in maintained (spontaneous) and in light-evoked firing rate. For these responses no obvious differences in effects of adenosine on the mentioned classes of ganglion cells were observed. The fact that some ganglion cells failed to respond to the neuromodulator is in accordance with the autoradiographic observation that only a subgroup of ganglion cells revealed adenosine labeling. The observation also corroborates the very consistent decrease in the ONR components under adenosine.

3.7. Controlled opening of the blood–retina barrier

To assess the physiological status of the BRB with its RPE- and "retinal vascular" components in the perfused cat eye we used both intraarterial dye injections and pharmacological compounds. Under normal perfusion of isolated eyes, the dyes Evans blue and lissamine green revealed intact retinal circulation without leakage, and there was no evidence for dye passage through the RPE (unpublished results). Two pharmacological experiments supported the notion that the BRB is tightly closed in the isolated perfused cat eye: the nicotinic antagonist gallamine triethiodide (Flaxedil®) administered intraarterially at 100 times the muscle relaxant dose had none of the effects on ERG and ONR that other nicotinic antagonists exerted (Jurklies *et al.*, 1996; Niemeyer, 1976a). Similarly, the putative retinal inhibitory neurotransmitter glycine at a concentration 1000 times higher than the threshold dose for isolated retina or under application using microiontophoresis failed to affect the light-evoked electrical signals (Schuurmans and Niemeyer, 1978). These results confirmed the notion that an intact BRB prevents gallamine and also glycine from reaching the retina.

An interest in opening temporarily the BRB arises in both experimental and therapeutic situations where the introduction of macromolecular or polar compounds into the central nervous system is desired. Among a number of substances suitable for producing a hyperosmotic effect, such as urea, arabinose, mannitol, and sucrose we chose the latter for practical reasons. Sucrose, available as a

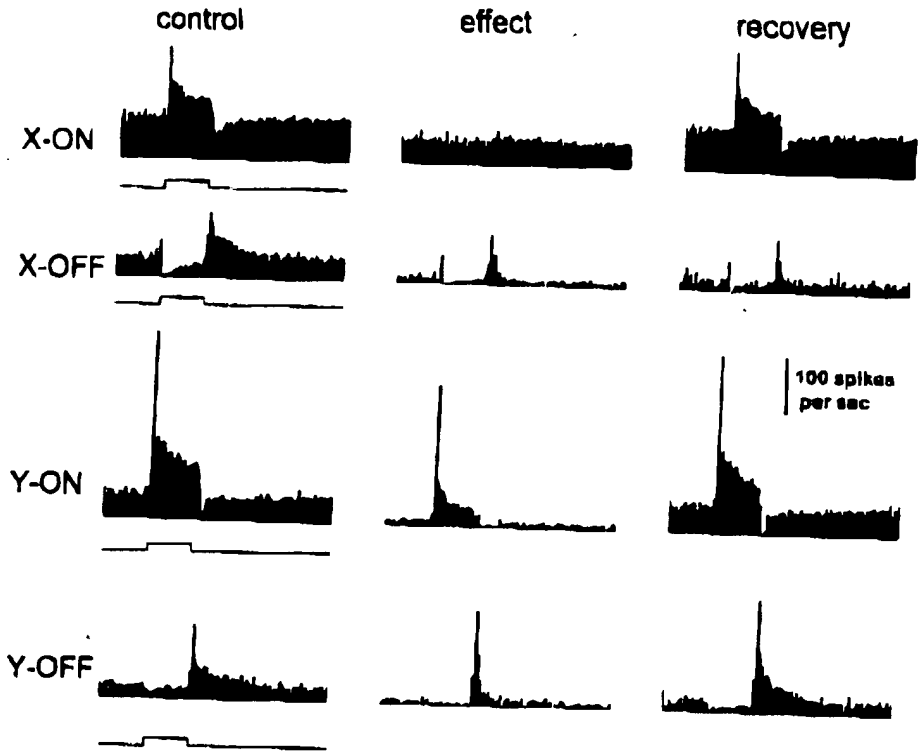

Fig. 12. Typical responses of four classes of ganglion cells to exogenous adenosine. Peristimulus-time histograms recorded before (control), during (effect) and after (recovery) application of intraarterial concentration of 4 μM adenosine. This concentration consistently affected vascular resistance, b-wave and ONR. The duration of the light stimulus (1 s, spot size 1.15°) centered over the receptive field of the respective ganglion cell is indicated by upward deflection of the bars below histograms (left column). Note the reduction, or even reversible extinction for the X-ONcenter cell (top row), of the light-responses and also a reversible reduction in maintained firing of all these cells (Macaluso, Frishman, Niemeyer, unpublished).

1.9 M solution was suitable to pump-inject into the perfusion system to reach the arterial concentration of 20–100 mM resulting in increases in osmolarity from 70 to 140 mmols above normal. We were interested in three aspects: (i) to determine the threshold for compromising the BRB in the cat eye, (ii) to demonstrate the opening of the BRB by showing accumulation of FITC-albumin within the retina, and (iii) to define electrical parameters that are likely to reflect the closed or (partially) opened status of the BRB. Preliminary data have been presented and I shall briefly outline relevant findings from this ongoing study (Kueng *et al.*, 1998; Kleinert *et al.*, 2000).

Immune-histochemical labeling of frozen sections of dissected retina from perfused cat eyes that had been exposed to brief (8–13 min) step increases in osmolarity revealed the following: FITC-albumin labeled, as expected, the choroidal and the retinal vasculature in all control- and experimental sections. Hyperosmotically-treated (70 mosmol and higher) preparations revealed the red label also on the apical side of the RPE, in the outer nuclear, outer plexiform and inner nuclear layers of the retina, but not or sparsely around the retinal vessels and capillaries. Sections of the *optic nerve* presented with comparable staining after the hyperosmotic challenge (Kueng *et al.*, 1998). We concluded that the RPE component of the BRB, as opposed to the retinal vessels, opened at least partly and evidently with a patchy distribution, whereas the retinal vessels failed to respond to this relatively small hyperosmotic step.

The light-evoked electrical signals, ERG b-wave, ONR and also the light peak of the DC ERG (in analogy to the clinical electrooculogram) were recorded before, during, and for 60 min after

the osmotic challenge (Kleinert *et al.*, 2000). Figure 13 depicts the results, including changes in the vitreo-scleral resistance assessed from pulses of constant current between intravitreal and scleral chlorided Ag-wire electrodes (see Section 2, and Fig. 3). We interpret the rapid and extensive changes in the amplitudes of the b-wave and of the ONR and the astonishingly rapid recovery as mainly *osmotic* effects on the ionic composition of the extracellular retinal environment. The optic nerve action potential was consistently less affected than the ERG b-wave (addressed also in Section 3.8). In contrast, the changes in the trans-retina/RPE/sclera resistance decreased to an entirely different extent and recovered slowly much like the light peak. The c-wave, recorded with pulses of light of 4 s duration, decreased rapidly, then changed to a slow P III waveform, recovering with a time course similar to that of the light peak, during and after the osmotic challenge. Both the light peak and the c-wave reflect the integrity and function of the RPE (Steinberg and Niemeyer, 1981; Steinberg *et al.*, 1985; Niemeyer, 1983; Dawis *et al.*, 1985)

The perfused mammalian eye preparation thus offers an opportunity to establish a threshold value of effective osmotic step changes for potential experimental and clinical applications when macromolecular substances should be directed to the brain or retina via intravascular injection. The injections can be systemic or more local by targetting the appropriate arteries. This route could be used to replace intravitreal or subretinal application.

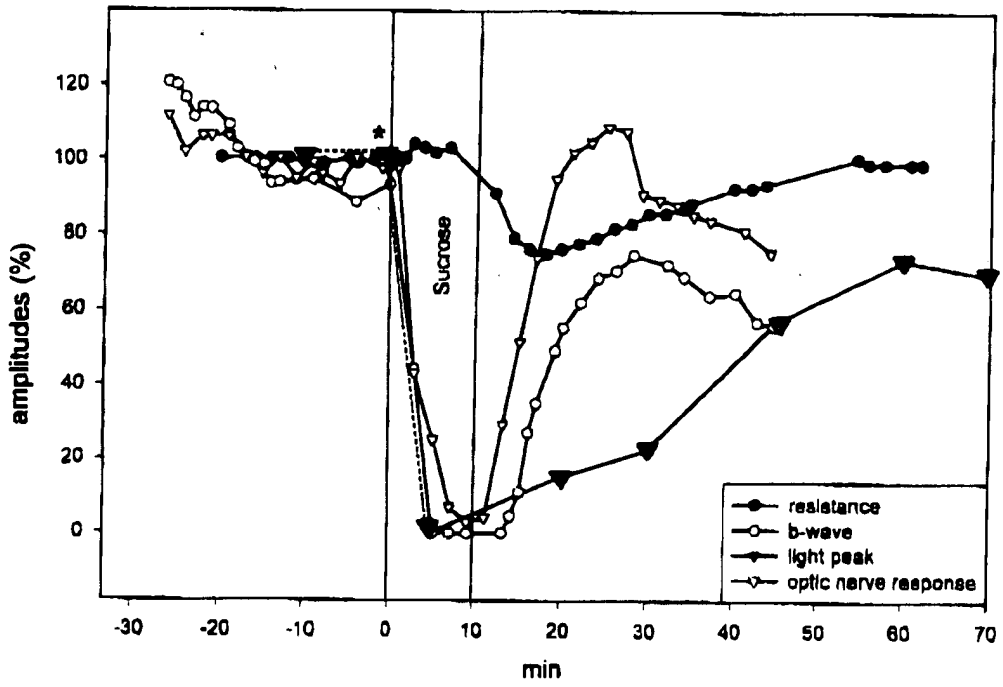

Fig. 13. Summary of results on hyperosmotic opening of the blood–retina barrier in perfused cat eyes. Fifty millimolar sucrose was applied by pump-driven injection for 10 min. Not all parameters could be recorded in every preparation. Typical changes over time in b-wave amplitude and optic nerve amplitude are shown as empty symbols. The vitreo–scleral resistance was measured by applying bipolar constant current pulses of 1 μA, 4 s in duration (filled circles), and light peaks (filled triangles), recorded at 40 min intervals were plotted as normalized amplitudes from several preparations. The dashed line indicates the connection from a fictive control immediately prior to the osmotic challenge (asterix) to the light peak recorded during the hyperosmotic phase. (The control cannot be recorded at time zero simply due to the > 10 min lasting light peak -changes in standing potential, which would be drastically distorted by the onset of hyperosmosis.) The signal amplitudes were taken as 100% before application of sucrose, and the changes during and after sucrose are indicated by percentage. Note the different time course of resistance and light peak changes compared to those of light-evoked ERG b-wave- and ONR amplitudes. The changes in light peak amplitude and in vitreo–scleral resistance appear to reflect the closed/open status of the BRB.

3.8. Differential effects on the ERG vs. optic nerve response: indicative of a non-neuronal contribution to the generation of the ERG b-wave

The contributions to the generation of the b-wave from non-neuronal elements, namely Müller cells (Newman and Frishman, 1991) are still an issue of debate (Shiells and Falk, 1999). The considerable body of experimental evidence for glial contribution to the b-wave generating mechanism comprises different techniques including current source density analysis, K_0^+ gradients along the axis of the Müller cell, localization of zones of high K^+ conductivity along the membrane of the Müller cell (Newman, 1987), morphology and electrophysiology in non-mammalian and mammalian species. It is generally accepted that the morphological and functional characteristics of Müller cells vary greatly among species, including distribution of K^+ conductance along the Müller cell axis and light-evoked radial currents (Newman and Frishman, 1991; Ripps and Witkovsky, 1985; Wen and Oakley II, 1990). A parallel between morphological and functional development has been reported in the chick retina: Rager (1979) observed a marked increase in surface area of Müller cells coinciding with the time of appearance of the b-wave. I support the notion that the extent of glial contribution to the generation of the b-wave is expected to vary greatly across species. Light-induced changes in the dark current along the radially oriented Müller cells depend on the proportion of the distal to the proximal increase in K_0^+ in relation to the gradient of K_0^+ and to the specific regional conductance (Dick and Miller, 1978; Oakley II *et al.*, 1992).

Based on a number of observations that revealed marked differences in variation of the b-wave amplitude compared to the variation in the optic nerve action potential, I would like to present novel indirect evidence for non-neuronal contribution to the generation of the b-wave (Niemeyer and Kuze, 2000). Masland and Ames III (1975) reported a dissociation between b-wave and ganglion cell activity in the isolated rabbit retina preparation with the optic nerve still attached. This is in accordance with our consistent observation that under conditions of inadequate retinal perfusion (e.g. due to partial leakage through severed arterial branches along the optic nerve, in the beginning of a perfusion experiment, or to transiently elevated intraocular pressure) the ERG is dominated by a slow P III with a small or non-detectable b-wave. The ONRs, however, are large under these conditions. As experiments in the perfused mammalian eye allow us to vary selectively one single variable, conditions are summarized in Table 3 that reduce the b-wave but do not affect optic nerve activity to the same extent. Table 3 also presents effects of adenosine that reveal clear differences in the extent of change in response magnitude of b-wave vs. ONR.

Effects of neurotransmitter-related agents can affect the b-wave and the ONR differentially based on the site of the corresponding receptors being located proximally to the site of the generation of the b-wave. That would not allow anything to be concluded with respect to non-neuronal contributions. However, some agents with binding sites in the inner retinal layers can exert divergent effects on the b-wave compared to the optic nerve: propranolol, a β-adrenergic antagonist, was shown to increase the rod-driven b-wave, but decrease the cone-driven b-wave, while a decrease in the plateau phase was a consistent finding in the ONR (Gerber and Niemeyer, 1988). The multiple effects of the neuromodulator adenosine (Section 3.6) seem to indicate that non-neuronal mechanisms increase the b-wave while the ONR was inhibited (Blazynski *et al.*, 1989).

Taken together, the marked differences between changes in b-wave and ONR under a variety of experimental conditions support the notion that the respective generating mechanisms are different. Ample evidence suggests that alpha amino adipic acid selectively damages Müller cells and reduces the b-wave in a broad range of species (see Zimmerman and Corfman (1984) for review). Our morphological finding of damage primarily to Müller cells by underperfusion as well as the observation of a preserved ONR in comparable experiments are indicative of a role of the retinal glia in generation of the b-wave in cat retina. The proportion of the glial contribution to the generation of the b-wave can be expected to vary greatly among mammalian species, and in comparison to lower vertebrates.

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ntraocular pressure) the
ow P III with a small or
ie ONRs, however, are
s. As experiments in the
allow us to vary selec-
conditions are summar-
ice the b-wave but do
vity to the same extent.
ects of adenosine that
the extent of change in
ave vs. ONR.
tter-related agents can
NR differentially based
onding receptors being
ite of the generation of
it allow anything to be
ion-neuronal contribu-
ts with binding sites in
exert divergent effects
to the optic nerve:
antagonist, was shown
b-wave, but decrease
hile a decrease in the
ent finding in the ONR
8). The multiple effects
lenosine (Section 3.6)
-neuronal mechanisms
ie ONR was inhibited

ed differences between
IR under a variety of
pport the notion that
chanisms are different.
it alpha amino adipic
lller cells and reduces
ange of species (see
(1984) for review).
of damage primarily
fusion as well as the
ONR in comparable
f a role of the retinal
ave in cat retina. The
bution to the genera-
pected to vary greatly
nd in comparison to

Table 3. Diverging responses of the b-wave compared to the ONR to induced experimental conditions

Experimentally induced change	Effect on b-wave amplitude	Effect on ONR ON-amplitude	Comment	Reference
Perfusion reduced to slow PIII only	Reduced to zero	Not measured	Histological changes, mainly in Müller cells	Remé and Niemeyer (1975)
Perfusion reduced by 33%	Reduction by 97%	Reduction by 52%	ONR less affected than b-wave	Niemeyer and Kuze (2000)
Transient elevation of intraocular pressure from 18 to 35 mmHg	Decrease in amplitude by about 50%	No change in amplitude	Marked immediate reduction in b-wave, while ONR unaffected	Kleinert and Niemeyer (unpublished)
Lowering glucose in perfusate	Stepwise reductions	Reduction	ONR less affected than b-wave	Macaluso *et al.* (1992a)
Low glucose plus insulin	Marked further reduction in amplitude; increase in latency	No further change in amplitude or in latency	Insulin under hypoglycemia affects b-wave but not ONR	Lansel and Niemeyer (1997)
Adding deoxyglucose 1.5, 2.5 or 3 mM	Graded decrease by up to 70%	Graded decrease by up to 30%	Drastic effect on b-wave, moderate effect on ONR	Niemeyer (unpublished)
Micromolar adenosine	Increase in rod-driven b-wave	Dose-dependent decrease in amplitudes	Increase in flow rate; opposite effects on b-wave vs. ONR	Blazynski *et al.* (1989)

4. PARALLELS TO PROBLEMS RELEVANT IN CLINICAL EYE RESEARCH

In vitro retina preparations in general and an isolated *intact* mammalian eye in particular lend themselves to study of the response threshold to very dim light, of the origin and of the generating mechanisms of components of the ERG. Physiological and pharmacological tools are usually used for this type of analysis. This approach can advance the understanding and the diagnostic potential of the various signal components (Fig. 11a) in clinical electroretinography (Berson, 1992; Fishman and Sokol, 1990; Zrenner, 1984; Niemeyer, 1997b, 1989a, 1991a; Niemeyer *et al.*, 1993; Robson and Frishman, 1999; Kellner *et al.*, 2000; G. Holder, this volume).

Direct access with the ERG reference electrode to the exposed sclera enabled Cringle *et al.* to correlate changes in b-wave amplitude with the location and extent of retinal lesions using arterially perfused dog eyes (Cringle *et al.*, 1986). It is therefore to be expected that the corneal ERG in patients would be affected regionally by retinal lesions that project electrical changes to the cornea rather than regarding the cornea as an equipotential structure.

Furthermore, mechanisms of actions of drugs — desired or evolving as side effects—can be assessed *in vitro* (Ripps *et al.*, 1989; Gerber and Niemeyer,1988; Uji *et al.*, 1988; Niemeyer, 1991a, 1998; Peachey *et al.*, 1993; Jurklies *et al.*, 1996).

A comprehensible characterization of the thresholds (Fig. 6) and dynamic ranges of the different components of the ERG (see Fig. 11a; Robson and Frishman, 1999) is of interest in phenotyping particularly with regard to retinal function in patients affected by retinal degeneration. This applies also to animal models for retinal degeneration, to knockout animals as models for human retinal degeneration and to assessment of therapeutic trials.

The glucose- and insulin-related studies, addressed in detail in Section 3.4, may contribute relevant mosaic pieces to clinical and experimental research on mechanisms of diabetic retinopathy. The restricted cellular distribution of glycogen in the rod- but not in the cone-pathway of the cat retina corroborated the physiologically identified sensitivity of rod-driven signals to changes in glucose. It remains to be shown if this pattern of localization of particulate glycogen holds for other mammalian and for the human retina. Also, the possibility to modulate reproducibly the quantity

of glycogen stored in the retina may be relevant for influencing glycogen stores in the human retina under critical conditions.

5. CONCLUSIONS

1. Advantages of an *in vitro* mammalian eye preparation include the synchronous electrophysiological monitoring of the RPE, the various layers of the retina and the optic nerve. Single-cell recordings are used to complement analysis of light-evoked field potentials. This monitoring can be used to study effects of one single variable — such as controlled arterial application of a substance — without extraocular regulatory influences.
2. The preparation is suited for short-term experiments (minutes to several hours). This time frame restricts the possibility of repeated intravitreal application of agents with slow diffusion to "pharmacologically dissect" the retina.
3. It has been shown that under adequate arterial perfusion an isolated mammalian eye can maintain structural integrity, physiological retinal performance, and — in recent results — sensitivity down to the range near human psychophysical thresholds.
4. The pharmacology of vascular dynamics can be studied quantitatively in perfused mammalian eyes because of the maintained integrity of the choroidal, retinal and ciliary circulation (Cringle *et al.*, 1997; Su *et al.*, 1995; Yu *et al.*, 1988).
5. Multiple studies in the perfused eye implemented controlled metabolic changes to elucidate pathophysiological mechanisms during induced hypoxia, changes in arterial glucose, as well as ischemia yielding novel information with potential clinical relevance.
6. Acute toxicity studies examining effects on RPE, retina and optic nerve can be done while assessing concurrently different levels of information processing.
7. Controlled opening of the BRB can be achieved with step increases in osmolarity, monitored by histological procedures and by specific electrical changes in standing potential, resistance and light evoked responses. Transitorily opening the blood–retina barrier may represent a conceivable technique to introduce retinal gene products for fast expression via vascular rather than intraocular injection.

6. FUTURE TRENDS

It is my hope that constructive applications will emerge from the techniques listed in Table 1 and from the details given in the "Methods". Future experiments will make use of the advantages of being able to control the arterial blood supply while recording directly from the retina, from the optic nerve and, when required, in combination with recordings of single-cell activity. The integrity of the retina together with the other structures of the eye *in vitro* facilitates a systemic approach allowing vascular, electrophysiological, and anatomical parameters to be investigated simultaneously. Light-evoked electrical field potentials can complement the recent studies characterizing subgroups of single ganglion cells according to the "weight" of their contributions to information processing. In this context, future studies on drug action and drug toxicity may also expand the use of mammalian perfused eye preparations.

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